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13. ABSTRACT (Maximum 200 Words) Breast cancer is the number one cause of death amongst cancer in women. New methods for early detection, diagnosis and treatment of cancer are always sought. In this annual report we present preliminary results on the isolation of antibody fragments (Fabs), isolated from phage display libraries, when affinity selected against breast cancer cell lines. Four rounds of affinity selection against a number of breast cancer cells lines and normal breast epithelial cells have been achieved. Monoclonal purified phage-Fab particles are being assessed for binding characteristics to the cells and isolated cellular materials. We also report on the initial stages of <i>in vivo</i> affinity selection where we will attempt to isolate Fabs that bind tumor material in tumor-bearing mice. So far we have concentrated on the procedures in normal mice. Tumor bearing mice are being generated and affinity selection against these will be carried out forthwith. Fab isolated will be assessed for binding characteristics and may be further mutated or engineering to incorporate radiometal atom binding sites for detection and imaging of primary tumors and metastases both <i>in vitro</i> and <i>in vivo</i> settings.				
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**Phage Fab Display Selection In Vitro and In Vivo: Novel Means to
Identify New Breast Cancer Avid Compounds**

Year 1 Annual Report

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Introduction

Breast cancer kills more American women each year than any other cancer (1). Breast cancer develops from normal breast epithelium through several stages. 'Invasive' breast carcinoma occurs when the epithelial cells invade the surrounding stroma (2) however the exact timing of the changing of the cells from benign to malignant remains unknown. Preinvasive lesions as well as some breast tumors may be detected by mammography, however many women with breast cancer remain undiagnosed using mammography alone. Alternative detection and diagnostic and therapeutic strategies are always sought. Phage display technology allows the isolation of moieties from a large and diverse starting library that bind to a given target. In our studies the target consists of either breast cancer cell lines or breast-derived tumors generated in a mouse model. The phage library used is one that displays a large number of unique antibody fragments (Fabs). The premise is that that Fabs may be isolated that target single proteins, molecular complexes or other unknown entities present on the breast cancer cells *in vitro*. Furthermore, by utilizing an *in vivo* model we may isolate Fabs that bind breast tumor material and at the same time remove non-binding phage or phage that bind normal body tissues and organs. After several rounds of affinity selection, phage of modest to high affinity for the target may be obtained. Isolated phage are sequenced to obtain the Fab sequence and soluble Fab may then be produced for further assessment of binding properties. Engineering of the Fab sequence also allows the incorporation of a radiolabeled atom that may be used to image and diagnose breast tumors and their metastases in an *in vivo* setting.

Body

The relevance of our proposed research is that affinity selection of Fab that bind breast cancer cells *in vitro* and tumor material *in vivo* will allow us to develop small tumor-avid molecules capable of targeting breast tumor material from a wide variety of sources. These Fab will be assessed as to their usefulness as diagnostic and imaging tools. Our first year progress is summarized below.

Aim 1: To isolate Fab molecules that bind to breast cancer cell surface antigens using phage display technology and breast tumor cell lines *in vitro*

We have the used the breast cancer cell lines MDA-MB435 and MCF-7 and a normal breast epithelial cell line, Hs578Bst as the target material in several rounds of affinity selection using a random CDR Fab phage-display library. In essence, cells were plated as normal in serum-containing media on small 30 mM tissue-culture plates. Approximately 10^{11} phage were added to each plate and incubated for 2-3 hrs at room temperature. Each of the three cell lines have different and visible gross morphology over the incubation period and this became a problem. Over the incubation and subsequent washing stages many of the cells would dislodge from the plates, especially in the case of the normal epithelial cell line. Thus, potentially we would be losing binding phage particles. To

overcome this we tried several different plating conditions including varying the type and brand of tissue culture plates used and varying the serum content of the plating media, however these did not seem to change the overall adherent properties of the cells.

In order to overcome this problem we varied the affinity selection conditions of the experiment. Cells were incubated with phage as normal for several hours. At the end of the incubation period, cells were dislodged from the plates either by pipetting the media up and down or by gentle scraping of the cells. The media containing cells were then added to a microcentrifuge tube and the cells pelleted at 3000g for five minutes. The supernatant was then removed. The supernatant in this case should contain non-binding and loosely binding phage and also any small cellular debris that was not pelleted. Fresh media or PBS was then added to the cell pellet and the pellet gently dislodged and agitated for 30 secs to one minute. Cells were then centrifuged and pelleted as before. Several rounds of washing were carried out and this number varied over subsequent rounds of affinity selection. This method of affinity selection and washing is quick and efficient however may suffer from the drawback that when cells are dislodged from the plates and are in suspension they will generally exhibit different morphology than when they are adhered to the plate. Proteins and molecular complexes present on the flattened adhered cells may not be present on the rounded cells in suspension. Potentially, we may lose phage particles that bind these targets. Also, washing by low-speed centrifugation and resuspension may still be a little stringent for the cell-phage binding interactions and potential weaker but specific binding phage may be lost, especially in the early rounds of affinity selection.

Isolation and Growth of Monoclonal Phage Populations

Following several rounds of affinity selection, individual bacterial colonies (>100, derived from individual phage) were picked, amplified and monoclonal phage populations produced. These phage populations were then assessed for binding characteristics to breast cancer cell line material.

Initial assessment of binding of phage populations against cell lines

With the observations of inadequate adherence of cells to plate surface we thought that using cells as the target ligand in an ELISA system would be problematic. This turned out to be the case and this method was quickly discarded. Instead we concentrated our efforts on devising an immunoblotting procedure to assess binding of our phage to cell line material. Cell suspensions were made and microlitre amounts (1-10 μ l) were dotted as small spots onto nitrocellulose membrane squares following by blocking of the membrane with tris-buffered saline containing 3% BSA. In individual containers, monoclonal phage populations were added (approximately 10^{10} - 10^{11} phage) and incubation proceeded for several hours. Following washing, a rabbit anti-phage antibody was added followed once again by washing before detection using a secondary anti-rabbit alkaline phosphatase conjugated antibody and appropriate colored precipitate detection system. This approach works extremely well when individual soluble proteins are used as the ligand. When cellular material is used it is a little more difficult. Due to the nature of the material used, the individual spots were visibly lumpy and the amount of material

added was difficult to keep consistent. Background signals were seen to be high and control incubations (with no phage added) still showed relatively large noise:signal ratio. Furthermore, the use of individual containers for each of the reactions was not only tedious but introduced potential variations in the incubation times, washing steps and final signal production stages.

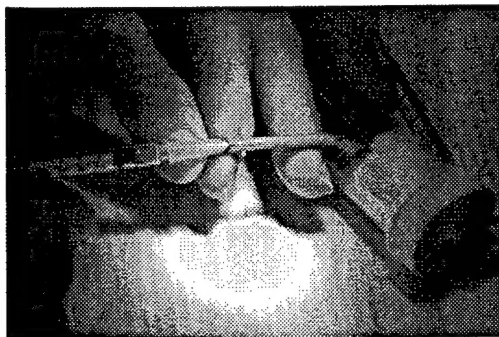
We are currently trying to assess whether we can spot phage onto the nitrocellulose and capture cellular material and/or proteins from a overlaid suspension. However, the secondary detection system for this will be difficult. If the phage manage to bind whole cells then we can detect these bound cells using an antibody to a common cell marker. However, if the phage bind a protein(s) that are individual in nature then secondary detection may be nigh on impossible since we will have no idea what that protein might be. Ways around these problems are currently being investigated.

We are also assessing whether proteins isolated from the cells may be subjected to SDS-PAGE, electroblotted to nitrocellulose membrane and detected with the phage. The disadvantage of this is that molecular complexes, dimmers etc will most likely be dissociated. However, by doing this we may be able to tell some information about the targeted proteins such as molecular weight for example.

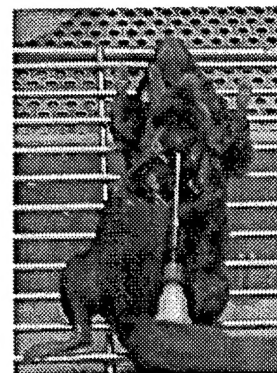
DNA derived from monoclonal phage populations is also currently being sequenced to determine the CDR sequence of the displayed Fab. This is being carried out using manual sequencing techniques and reading of sequences and data is still in the process of being analyzed. Sequence analysis will hopefully yield a consensus sequence(s) which generally indicates a successful affinity selection against a target(s).

Aim 2: To isolate Fab molecules that bind breast tumors and their metastases using phage display technology utilizing an *in vivo* strategy

In the initial phase of *in vivo* affinity selection we focused on mock selections using a normal mouse. Normal healthy CF1 mice were injected via the tail vein with 1×10^{12} and the phage allowed to circulate through the blood system for one hour. Following this, the mouse was perfused with warm tissue culture fluid for several minutes via the heart. The perfusion step allowed the removal of many blood-system associated phage that would confound the calculation of number of phage binding to each organ, since the organs are highly vascularized by blood vessels. Organs were removed, washed and binding phage eluted for titrating.



Injection of Phage via Tail Vein



Perfusion of Warm Media via Heart and Blood System

A typical experiment gives phage titers similar to those below.

Tissue	Titer (cfu/ml*)	Tissue Mass (g)	Titer/g Tissue (cfu/g)
Kidney	5.5E+07	0.177	3.1E+08
Bladder	5.5E+05	0.046	1.2E+07
Heart	5.5E+06	0.11	5.0E+07
Brain	5.5E+07	0.133	4.1E+08
Intestines	6.0E+04	0.154	3.9E+05
Pancreas	5.5E+06	0.114	4.8E+07
Liver	5.0E+09	0.341	1.5E+10
Blood	1.0E+12		

*: cfu – colony forming units

It is evident that vast majority of phage particles bind or are trapped in the blood system and in the liver, brain and kidney. Now that we have established the selection protocol in normal mice we are currently inducing tumors in mice using MDA-MB 435 cells. Once these tumors are established we will start the affinity selection protocols. When using affinity selection in the tumor bearing mice we will also isolate mammary tissue, lymph nodes, lung tissue and any visible metastases. Metastases may have different cell markers than primary tumor masses and thus may yield valuable binding phage.

Key Research Accomplishments

1. Four rounds of affinity selection against breast cancer cell lines and normal breast epithelial cells achieved
2. >100 monoclonal phage populations isolated and currently being sequenced at the DNA level for the random CDR sequence
3. Assessment of binding characteristics of phage populations in progress
4. *In vivo* distribution of phage in normal healthy mice assessed and proof of principle that phage may be isolated and amplified from target tissues

Conclusions

Using affinity selection in an *in vitro* setting with breast cancer cell lines was held back due to technical difficulties with plating and adherence properties of the cells used. This has been overcome using a modified affinity selection procedure and we have successfully completed four rounds of affinity selection. Assessing the binding characteristics of the isolated phage populations remains a difficult task. Large numbers of phage need to be screened and since the target molecules (the cells) actually consist of a multitude of potential targets, screening must be carried out using whole cells or the proteins derived from these cells. We are currently investigating appropriate methods of screening these phage.

The *in vivo* affinity selection still remains an excellent approach. We have shown that, within an hour, phage travel to all parts of the body, we can isolate these phage

particles and we can reamplify them. Tumor-bearing mice are being generated and the affinity selection procedures will start forthwith.

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